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Studies on the Mechanism of the Bacterial
Metabolism of Nicotine and Related Compounds

Parts of the investigations conducted during 1957-58 have been described in the semi-annual report of June 9, 1958 and in two papers which will appear in the Journal of Biological Chemistry in January 1959. In addition, two papers are being prepared covering other aspects of the year's work. Consequently, this report will be brief, and the above mentioned semi-annual report and papers should be considered part of this annual report.

During the last half-year's work, investigations have yielded further information on the properties of the hydroxylating enzyme, the second oxidative product, and the blue pigment. These topics will be dealt with in turn.

Specificity of the hydroxylating enzyme

It had previously been shown that an enzyme fraction which precipitates from a crude extract between 20 and 40 percent ammonium sulfate saturation, hydroxylates and oxidizes nicotine to 6-hydroxynicotine. The same fraction was checked for its activity on a variety of compounds structurally related to nicotine. Pyridine, nicotinic acid, nicotinamide, anabasine, and 6-hydroxy-3-succinoylpyridine were not oxidized, but nor-nicotine and myosmine were oxidized with the consumption of 0.5 micromole of oxygen per micromole of substrate. With myosmine, the ultraviolet absorption maxima at 223 and 264 millimicrons changed to a single maximum at 298 millimicrons as a result of oxidation. The latter absorption maximum is characteristic of 6-hydroxymyosmine, thus leading to the conclusion that this compound was formed during the single step enzymatic oxidation. After the oxidation of nornicotine, the absorption maximum at 260 millimicrons disappeared and two new maxima at 232 and 300 millimicrons appeared. This change in the absorption spectrum is parallel to what occurs in the oxidation of nicotine to 6-hydroxynicotine; the 260 millimicron peak disappearing and peaks at 232 and 295 millimicrons appearing. On the basis of the observed spectral changes it can be tentatively concluded that 6-hydroxynornicotine is the product of oxidation. However, in this instance there is no data available in the literature for comparative purposes.

Thus, the enzyme hydroxylates certain compounds closely related to nicotine at the 6 position of the pyridine ring. It was this finding

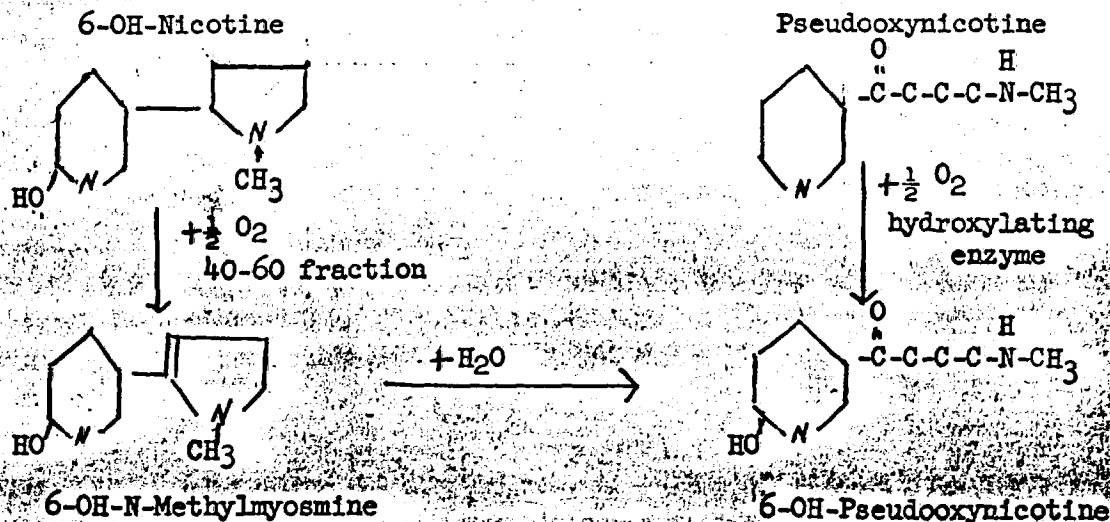
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that ultimately allowed a conclusive identification of the second oxidative product of nicotine (see below). Not too much can be said concerning the structural requirements of the enzyme. The hydroxylation of myosmine indicates that the N-methyl group of nicotine is not a necessary structural feature. Even though pseudooxynicotine is also oxidized (see below) this does not rule out the intact pyrrolidine ring as a requirement since ring closure could occur prior to oxidation. It is also apparent that this enzyme is not related to the hydroxylating enzyme involved in nicotinic acid oxidation, even though in the latter case it is also the six position of the pyridine ring that is attacked.

Identification of the second oxidative product of nicotine

Data presented in the semi-annual report (1958) suggested that the second oxidative product of nicotine degradation was 6-hydroxy-N-methylmyosmine. Elemental analyses of the enzymatically produced product indicated that it was isolated as a monohydrochloride salt. The product was metabolized by both resting cells and crude enzyme extracts in a manner consistent with its role as a true intermediate in nicotine degradation. Unfortunately, 6-hydroxy-N-methylmyosmine has not been described in the literature, and no information is available for comparative purposes. Thus a conclusive identification of the product by classical criteria is not immediately possible. Preliminary attempts to synthesize 6-hydroxy-N-methylmyosmine were unsuccessful; further efforts in this direction are presently underway. Fortunately, another approach to identification was possible.

Taking advantage of the spectrum of action of the hydroxylating enzyme, and its specificity for the six position, the oxidation of pseudooxynicotine by this enzyme was attempted. Oxidation occurred with the uptake of 0.5 micromole of oxygen per micromole of substrate, and yielded a product which, in the reaction mixture, had a single absorption peak at 290 millimicrons under acidic conditions. Under alkaline conditions, the peak shifted to a new maximum at 310 millimicrons, and the ratio of the two absorption maxima (alkaline/acid) was 2.16. The second oxidative product of nicotine degradation, formed by the single step oxidation of 6-hydroxynicotine, has identical absorption characteristics. Since it can be assumed, from the data presented in the previous section, that pseudooxynicotine was hydroxylated in the six position, the reactions with the two substrates can be formulated as follows:



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These data, combined with the previously available information establish 6-hydroxy-N-methylmyosmine (and/or its hydrated form, 6-hydroxypseudooxynicotine) as the second oxidative product in nicotine metabolism. There are some aspects of the chemistry involved that are still not clear. It is not known whether the hydrolytic step shown in the reaction sequence is an enzymatic one or not. It is possible that 6-hydroxy-N-methylmyosmine and 6-hydroxypseudooxynicotine are in spontaneous equilibrium under physiological conditions and that the acid base shift in ultraviolet absorption is due to the interconversion of the open and closed forms.

The blue pigment

If the 45-60 percent ammonium sulfate enzyme fraction is allowed to act on 6-hydroxymethylmyosmine in the presence of methylene blue or brilliant cresyl blue, oxygen is consumed and the substrate disappears. This disappearance is accompanied by the appearance of a new absorption maximum at 353 millimicrons. Under the same conditions, but in the absence of dye, no detectable oxygen uptake occurs, and a blue pigment that is apparently identical to the pigment formed during the growth of the bacterium on nicotine appears in the reaction mixture. No pigment is formed when the same reaction mixture is incubated under anaerobic conditions. Thus oxygen appears necessary for pigment formation although it does not appear to be consumed during the reaction.

In order to study this phenomenon further, an effort was made to obtain a quantitative measure of pigment formation. Using pure, crystalline 6-hydroxynicotine as the substrate and the 45-60 fraction as the enzymatic material, reactions were run in the absence of dye over a range of substrate concentrations between 0.01 and 0.04 micromoles per two ml. Production of pigment was followed spectrophotometrically by measuring absorbance at 600 millimicrons. When absorbance reached a maximum and then remained constant with time, it was assumed that the substrate had been completely consumed. This was confirmed by failure to detect 6-hydroxynicotine or 6-hydroxymethylmyosmine spectrophotometrically in such reaction mixtures. Assuming a quantitative conversion of substrate to pigment, a molar extinction coefficient for the latter was calculated and used to determine the amount of pigment formed in other experiments. On the basis of several such experiments, the following information was obtained:

(1) the pigment has a very high molar extinction coefficient, of the order of 5300. This means that very intensely colored solutions can result from minute quantities of pigment.

(2) the rate of pigment formation, under conditions where substrate was not completely consumed during the course of the experiment, decreased markedly with time. For example, starting with 2.6 micromoles of 6-hydroxy-methylmyosmine, only 0.36 micromole of pigment was formed in two hours using 2.5 mg. enzyme protein. From such results, it was apparent that conventional manometric techniques are not sufficiently sensitive to determine whether oxygen is consumed during pigment formation. If one

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assumes that one-half micromole of oxygen is taken up per micromole of substrate converted to pigment, then a total of only 4 microliters of oxygen would have been used under the conditions of the experiment described. This would not be detectable with the conventional Warburg apparatus and no instrument of greater sensitivity is available to us.

In another series of experiments, the rate of pigment formation was compared in reaction mixtures incubated anaerobically previous to exposure to air with mixtures shaken in air from the start. It was found that an anaerobic incubation resulted in a more rapid rate of pigment formation once air was introduced. Further, although no pigment was formed anaerobically, a new compound that differed from 6-hydroxymethylmyosmine and the third oxidative product of nicotine was detected chromatographically and spectrophotometrically. These data show that one or more intermediates exist between 6-hydroxymethylmyosmine and pigment, and are consistent with the previously postulated intermediate described as compound X in the semi-annual report. It is possible that this anaerobic step is the hydrolytic removal of methylamine from the molecule. This hypothesis is being investigated currently, concomitant with the studies on the third oxidative product.

Attempts to isolate the pigment in pure form have not succeeded. Analyses on the best two samples so far obtained showed that the product as isolated was grossly contaminated with inorganic materials. We are continuing our efforts to obtain a pure product.

Once formed the pigment was found to be metabolically inert. Thus when pigment formation from second product was allowed to occur in the absence of dye and then dye was tipped into the system to allow oxidation of residual substrate, the amount of pigment remaining at the end of oxidation was unaltered.

Speculations on the chemical nature of the pigment are premature and the complete elucidation of its structure is probably beyond the scope of this investigation. It does appear to us however that the pigment must be at least a dimer of some base unit derived from 6-hydroxymethylmyosmine since no simple alteration of the second product either by the addition of water or by the uptake of a small amount of oxygen can be conceived that would yield such an intensely pigmented compound. Our data do show clearly, however, two points of importance in the relation of pigment formation to nicotine metabolism: the pigment is formed between the second and third oxidative step; and that it is a side product and not an intermediate in nicotine degradation.

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